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Development of next-generation fluorescent turn-on sensor to simultaneously detect and detoxify mercury in living samples

Abdul Malek[†, Φ], *Kallol Bera*[#, Φ], *Shrutidhara Biswas*[§], *Govindaraj Perumal*[¥], *Anand Kant Das*[€], *Mukesh Doble*[¥], *Tiju Thomas*[*‡] *and Edamana Prasad*[*†]

[†]Department of Chemistry, Indian Institute of Technology Madras, Chennai-600036, India.

[#]Chemical Sciences Division, Saha Institute of Nuclear Physics, 1/AF Bidhannagar, Kolkata 700064, India.

[§]Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati-781039, India.

[¥]Department of Biotechnology, Indian Institute of Technology Madras, Chennai-600036, India.

[€]Department of Chemical Sciences, Tata Institute of Fundamental Research, Mumbai- 400005, India.

[‡]Department of Metallurgical and Materials Engineering, Institute of Technology Madras, Chennai-600036, India.

ABSTRACT

Strategies for simultaneous detection and detoxification of Hg^{2+} using a single sensor from biological and environmental samples are limited and have not been realized in living organisms so far. We report a highly selective, small molecule 'turn-on' fluorescent sensor, PYDMSA, based on the cationic dye, Pyronin Y (PY), and chelating agent, meso-2, 3-dimercaptosuccinic acid (DMSA) for the simultaneous detection and detoxification of inorganic mercury (Hg^{2+}). After Hg^{2+} detection, concomitant detoxification is carried out with sufficient efficacy in living samples which makes the sensor unique. PYDMSA exhibits high selectivity for Hg^{2+} over other competing metal ions with an experimental detection limit of ~300 pM in aqueous buffer solution. When PYDMSA reacts with Hg^{2+}, the CS-C^9 bond in the sensor gets cleaved. This results in the 'turn-on' response of the fluorescence probe with a concomitant release of one equivalent of water-soluble Hg^{2+}-DMSA complex which leads to synchronous detoxifying effect. The sensor by itself is non-toxic to cells in culture and has been used to monitor the real-time uptake of Hg^{2+} in live cells and zebrafish larvae. Thus, PYDMSA is a unique sensor which can be used to detect and detoxify mercury at the same time in living samples.

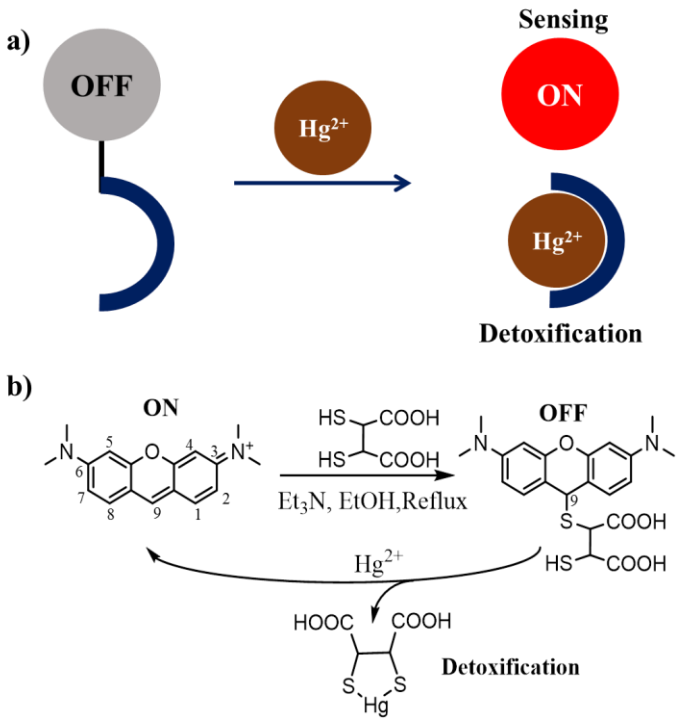
INTRODUCTION

Mercury is one of the highly toxic, bio-accumulating, and hazardous heavy metals having adverse effects on various life forms. These adverse effects include enhanced risk for severe brain damage, kidney problems, immune dysfunction and motion disorders in humans.[1-4] The World Health Organization (WHO) and the United States Environmental Protection Agency (USEPA) have prescribed stringent limits regarding the maximum contamination level for mercury in drinking water. There have been concerted efforts to develop sensitive methods for detecting mercury at low levels in environmental and biological samples.[5-7] These include (a) analytical techniques such as inductively coupled plasma optical emission spectrometry (ICP-OES) with detection limit of 0.06 μg[8], cold vapor atomic absorption spectrometry (CV AAS) which reduces Hg^{2+} to elemental mercury Hg(0) and detects the atomic absorption signature of Hg with a detection limit of 0.7ng[9] and inductively coupled plasma mass spectrometry (ICP-MS) with a detection limit of 0.001 ppb[10,11] (b) fluorescent and colorimetric sensors,[5] (c) surface-enhanced Raman spectroscopy (SERS) based sensors[12] (d) ratiometric sensors,[13] (e) photoelectrochemical sensors,[14] (f) label-free sensors,[15] (g) micro-electromechanical sensors based on surface acoustic wave and quartz crystal microbalance,[16] (h) naked eye sensors[17] (i) reusable DNA-functionalized hydrogels[18] and surfaces[19] and others.[20-24] We have earlier developed a rhodamine−rhodanine based 'turn-on' fluorescent sensor (RR1) for real-time monitoring of inorganic mercury (Hg^{2+}) uptake in cells and zebrafish larvae.[25] We were inspired to take the next step of developing simple and elegant strategies to simultaneously detect and detoxify Hg^{2+} from biological samples with high sensitivity and efficacy.

There have been sporadic attempts in the past for simultaneous detection and detoxification of Hg^{2+} from water samples and cells. Interesting strategies such as surface-displayed system in

mercury-resistant strains of *E.coli*[26], luminescent metal-organic framework[27], fluorescent thioether based organic framework[28] and pillararene-based aggregation induced supramolecular system[29] to detect and remove Hg^{2+} from water samples have been reported. While in cells, methods involving liposome system that encapsulated fluorescein for detection of Hg^{2+} (up to 10 nM) and chelating agents such as meso-2,3-dimercaptosuccinic acid (DMSA) for detoxification[30] or 7-N-diethyl coumarin and DMSA based fluorescent theranostic agents[31] have been used. However, the strategies for simultaneous detection and detoxification of Hg^{2+} from biological and environmental samples is limited and has not been realized in living organisms so far.

Herein, we develop a 'turn-on' fluorescent sensor, PYDMSA, based on a visible dye, Pyronin Y, and a rather routinely used Hg^{2+} chelating agent, meso-2,3-dimercaptosuccinic acid (DMSA).[32] We speculate that introducing the DMSA to Pyronine Y would (i) increase water solubility of PYDMSA due to the presence of two –COOH (pKa = 3.5) groups which will form Na^+ or K^+ salt in the buffer medium, (ii) increase its affinity for Hg^{2+} in competitive aqueous media via anchoring of 'soft' metal ion Hg^{2+} through the two thiol groups, (iii) lead to its quick fluorescence and color responses (i.e., facilitate real-time detection) through subsequent cleavage of the CS-C^9 bond by coordinating with Hg^{2+}, (iv) improve selectivity, and (v) detoxify Hg^{2+} through the formation of water-soluble Hg^{2+}-DMSA complex instead of the formation of insoluble toxic HgS in live cells[29] as suggested in the plausible sensing and detoxification mechanism in **Scheme 1**.



Scheme 1. (a) Design of the sensor (PYDMSA). We speculated that introducing the DMSA to Pyronine Y would provide a platform where Pyronin Y will help in fluorescent turn off-on characteristics, and DMSA will form a chelating complex with Hg^{2+} for the detoxification. **(b)** Synthetic route and plausible sensing mechanism of PYDMSA. The CS-C^9 bond in the sensor gets cleaved when PYDMSA reacts with Hg^{2+}, resulting in the 'turn-on' of the fluorescence with the concomitant release of one equivalent of water-soluble Hg^{2+}-DMSA complex for synchronous detoxifying effects.

EXPERIMENTAL SECTION

Pyronin Y, meso-2,3-dimercaptosuccinic acid (DMSA), Triethyl amine and all other starting materials, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and CCK-8, are obtained from Sigma-Aldrich, USA. All the materials are used as received. UV grade solvents are purchased from Spectrochem Pvt. Ltd. ^1H and ^{13}C NMR are collected on a Bruker 400 MHz spectrometer (^1H, 400MHz, ^{13}C, 100 MHz). Mass spectra for the compounds are recorded using a Micromass Q-TOF mass spectrometer. UV-Vis absorption experiments are carried out using a Jasco V-660 spectrophotometer. Fluorescence measurements are carried out

on a Horiba Jobin Yvon Fluoromax-4 fluorescence spectrophotometer. FT-IR spectra for the sensor are recorded using Jasco FTIR-4100 spectrophotometer at room temperature. Absorbance for toxicity assay was measured using the microplate reader (Enspire, Perkin Elmer, USA). Confocal imaging experiments were carried out using a Carl Zeiss LSM 710 confocal system with 543 nm laser.

The probe PYDMSA was synthesized in ~ 4 hours from the reaction of Pyronin Y (1 eqv.) and meso-2,3-dimercaptosuccinic acid (1 eqv.) in the ethanolic medium at 80 °C. Triethyl amine (4 eqv.) was used as a base. The probe can be synthesized in one step with 50% yield (**Scheme 1b**). The probe was purified by column chromatography using DCM:MeOH (99:1) as eluent. PYDMSA is stored at –20 °C to avoid decomposition.

RESULTS AND DISCUSSION

PYDMSA is further characterized by Fourier transform infrared spectroscopy (FTIR) (**Figure S1**), nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) (**Figure S2**), and high-resolution mass spectrometry (HRMS) (**Figure S3**). CS-C^{9} vibration is observed around 660 cm^{-1} in the FTIR spectrum of PYDMSA which confirms the formation of the molecule through the introduction of CS-C^{9} bond. The exact mass is found to be 450.12 which is consistent with the proposed structure. In aqueous medium, the sensor is colorless and shows strong absorption maximum around 390 nm in phosphate buffer saline (PBS) at pH 7.4 due to the lack of long conjugation (**Figure S4**). Upon addition of Hg^{2+}, the sensor exhibits a marked color change, from colorless to pink (**Figure S5**), and can be easily detected with the naked eye at and above 2 μM of Hg^{2+}. PYDMSA also responds rapidly towards Hg^{2+} within 0.1s of its addition (**Figure S6)**. Further, addition of Hg^{2+} results in a strong absorption maximum around 550 nm and the absorption

maximum near 390 nm almost disappears (**Figure S4**). As soft-soft interaction is more favored over hard-soft interaction, Hg^{2+} being 'soft' metal ion shows a very strong affinity to 'soft' mercapto (-SH) group. Hg^{2+} ion interacts with sulfur atoms of two mercapto moieties and forms coordination complex which results in CS-C[9] bond breaking. This further leads to the formation of Hg^{2+}-DMSA complex which gets separated from the PY (**Scheme 1b**). Thus the PY moiety gets back its long conjugation and exhibits lower energy characteristic absorption maximum (λ_{ex}^{max}) at 550 nm and corresponding emission (λ_{em}^{max}) near 570 nm. High-resolution mass spectrometry (HRMS) shows a peak around 378.3 which corresponds to the Hg^{2+}-DMSA complex (calculated mass: 378.77), thus, providing direct evidence for its presence. This, in turn, releases pyronin Y (observed mass: 302.80, calculated mass: 302.09) in the solution (**Figure S7**).

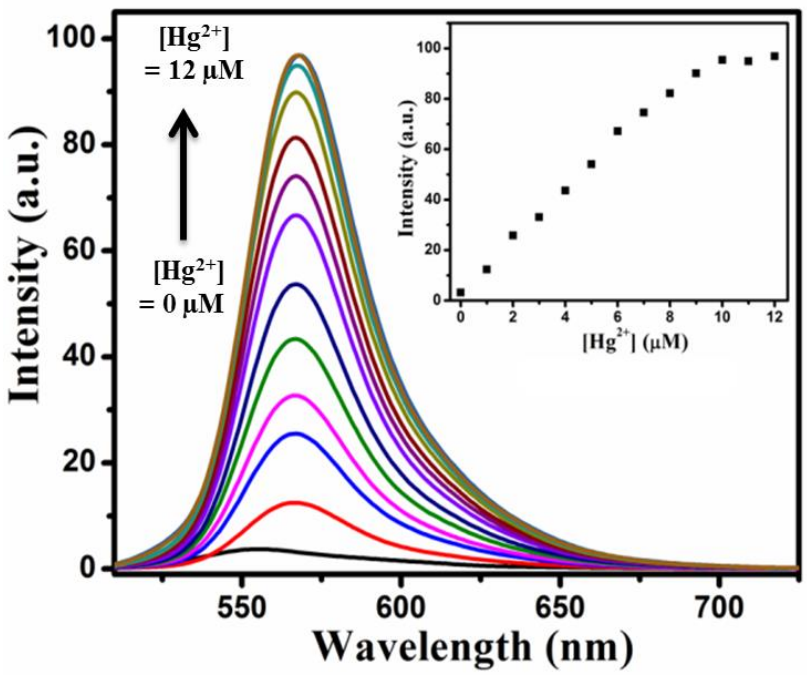


Figure 1. Fluorescence response of PYDMSA (10 µM) upon addition of Hg^{2+} (0-12 µM) at 25 °C in PBS (pH 7.4). The excitation wavelength (λ_{ex}) was 490 nm. The plot indicates an emission peak (λ_{em}) at 570 nm such that the peak intensity gradually increases with increasing [Hg^{2+}]. Inset shows the plot for fluorescence intensity with increasing [Hg^{2+}].

It is observed that upon increasing the concentration of Hg^{2+}, the fluorescence intensity of PYDMSA increases linearly. The fluorescence intensity of PYDMSA is plotted against varying concentrations of Hg^{2+} (0-12 μM) in PBS at pH 7.4 (**Figure 1**). Linear behavior (Correlation coefficient $R^2 = 0.99$, Figure S8) of the intensity with the increasing Hg^{2+}concentration is evident from these plots. The Job's plot indicates 1:1 stoichiometry of complexation between the PYDMSA and Hg^{2+} (**Figure S9**). Hence, PYDMSA can be used for stoichiometric detection of Hg^{2+}. The limit of detection (LOD) can be experimentally determined by carrying out the fluorescence titration experiment starting with a very low concentration of Hg^{2+}. We observed that the sensor is capable of detecting Hg^{2+} as low as ~300 pM in aqueous buffer solution (**Figure S10).** The lowest experimental detection value of [Hg^{2+}] by PYDMSA is far below the safe limit of Hg^{2+}contamination level in drinking water given by USEPA, which is 2 ppb (~10 nM). This makes PYDMA a sensitive probe with potential practical implications. Furthermore, specificity of Hg^{2+}-PYDMSA interaction is explored. The fluorescence response of 10 μM of PYDMSA in the presence of 10 μM of various metals ions (Fe^{3+}, Al^{3+}, Cr^{3+}, Fe^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Ag^{+} Na^{+}, K^{+} etc.) was examined under identical conditions. A 25 fold enhancement of fluorescence in the presence of Hg^{2+} is observed while other metal ions have showed a negligible change in fluorescence (**Figure 2**). Moreover, addition of higher concentrations (50 μM) of interfering ions to PYDMSA (5 μM) (**Figure S11**) also has indicated a high selectivity of Hg^{2+} towards PYDMSA.

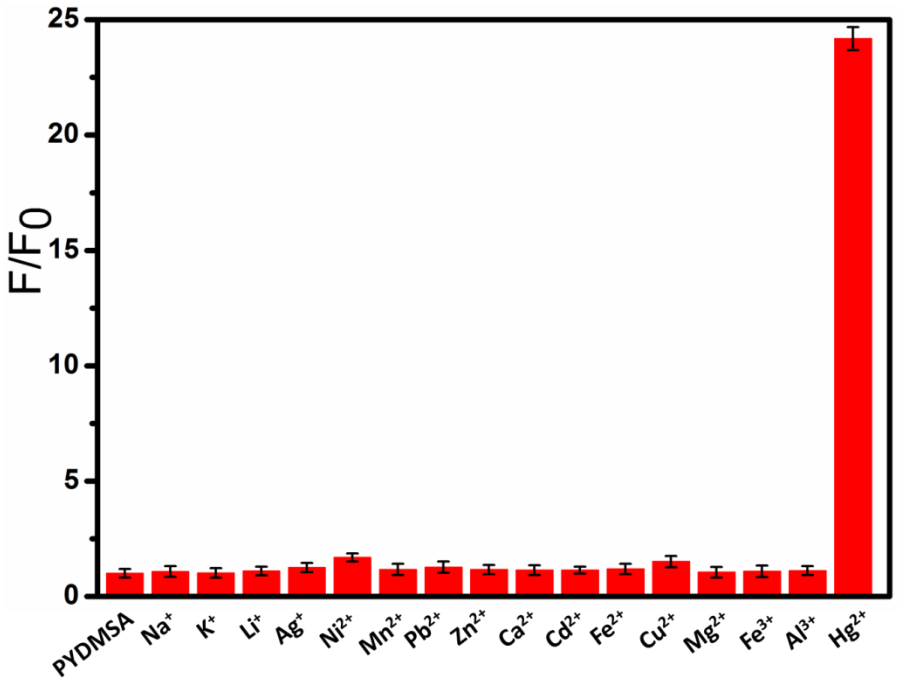


Figure 2. Relative fluorescence intensity changes after addition of 10 μM of various metal ions to 10 μM PYDMSA.

Owing to its chemical and spectroscopic properties; PYDMSA would be permeable to cells and would be ideally suited for monitoring and detoxifying Hg^{2+} ions in various kinds of living samples such as in cells and model organisms like the zebrafish larvae. To test this, we monitored the real-time uptake of Hg^{2+} in two different cell types; HEK293T (somatic cell) and RN46A (neuronal cell line), and in zebrafish larvae in the presence of PYDMSA. The HEK293T and RN46A cultured cells were treated with 5 μM of PYDMSA for 20 mins followed by exposure to 5 μM of $HgCl_2$ for 20 min at 37°C. Fluorescence microscopic images of the cells subjected to PYDMSA alone and subsequent incubation with Hg^{2+} are shown in **Figure 3.** We observed a marked increase in the fluorescence intensity of PYDMSA in presence of Hg^{2+} in both the cell types. Furthermore, upon incubation of sub-lethal dose of 10 μM of Hg^{2+} with the

zebrafish larvae pre-exposed to 10 μM of PYDMSA showed a similar increase in fluorescence as observed in cells (**Figure 3g and 3h**).

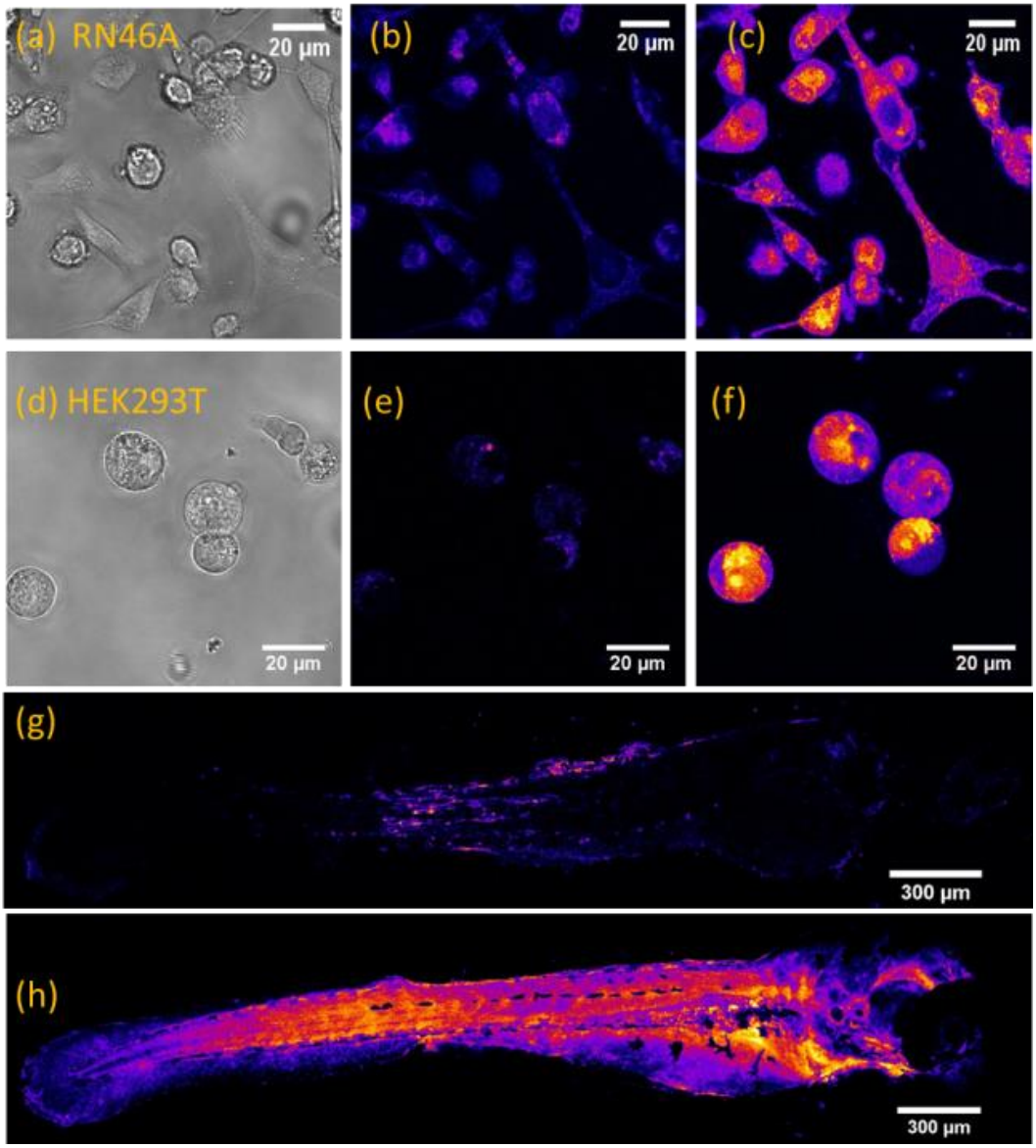


Figure 3. Monitoring the uptake of Hg^{2+} using PYDMSA in live cells and zebrafish larvae. (**a**) and (**d**) are the bright field transmission image of RN46A and HEK293T cell respectively. (**b**) and (**e**) are the fluorescence confocal images of RN46A and HEK293T cell respectively treated with PYDMSA (5 μM). (**c**) and (**f**) are the fluorescence confocal image of RN46A and HEK293T upon subsequent exposure to 5μM of Hg^{2+} for 20 mins. λ_{ex} = 540 nm. (**g**) and (**h**) are the fluorescence confocal image of a live 5-day-old zebrafish treated with PYDMSA alone (10 μM) and subsequent exposure to Hg^{2+} (10 μM) for 20 minutes.

The real-time uptake experiments suggest that PYDMSA can penetrate the tissue of a live zebrafish with ease. The efficacy of a sensor lies in its ability to penetrate the tissue at the whole organism level. In this context, PYDMSA is capable of penetrating even the blood-brain barrier and spreads uniformly over all tissue regions.

Interestingly, the water-soluble Hg^{2+}-DMSA complex would result in the simultaneous detoxification step which should then lead to increased viability in the cell death assays. To verify this hypothesis, the cell viability at different concentrations of Hg^{2+} and the PYDMSA was tested in cultured mouse fibroblast L929 cells and assessed using the cell counting kit (CCK-8). The samples (Hg^{2+} alone, PYDMSA alone, a mixture of Hg^{2+} and PYDMSA; in case of co-incubation Hg^{2+} was incubated first for 1 h followed by PYDMSA) were solubilized in Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium to get the desired final concentrations. L929 fibroblasts cells with a seeding density of 1×10^4 cells were plated in a 96-well cell culture plates at 37 ^oC with 5 % CO_2 for 24 hours. The cells were then treated with 200 µL of various concentrations of the samples. The untreated wells with the culture medium alone were used as control samples. After 24 hours of treatment, the CCK-8 reagent was added to each of the wells (treated and control) and incubated for 3-4 hours. Absorbance was measured at 450 nm. From the cell viability assay (**Figure 4**), it was found that Hg^{2+}alone at 25 µM for 24 h is toxic to cells (57 ± 4, cell viability ± SD). This cell viability was lower at 50 µM concentration of Hg^{2+} (32 ± 1) when compared to 25 µM concentration. PYDMSA alone appeared benign to the fibroblast cells (99 ± 8 at 25 µM; 102 ± 5 at 50 µM). This makes it highly useful even at higher concentrations in biological samples. Intriguingly and in agreement with our expectations, when PYDMSA was added to the Hg^{2+} treated cells, the toxic effects of Hg^{2+} was substantially neutralized which is reflected in the increased cell viability (100 ± 9 at 25 µM Hg^{2+}+PYDMSA;

83 ± 6 at 50 µM Hg^{2+} +PYDMSA). All the cell viability data were normalized to untreated control cells whose viability was assumed as 100%.

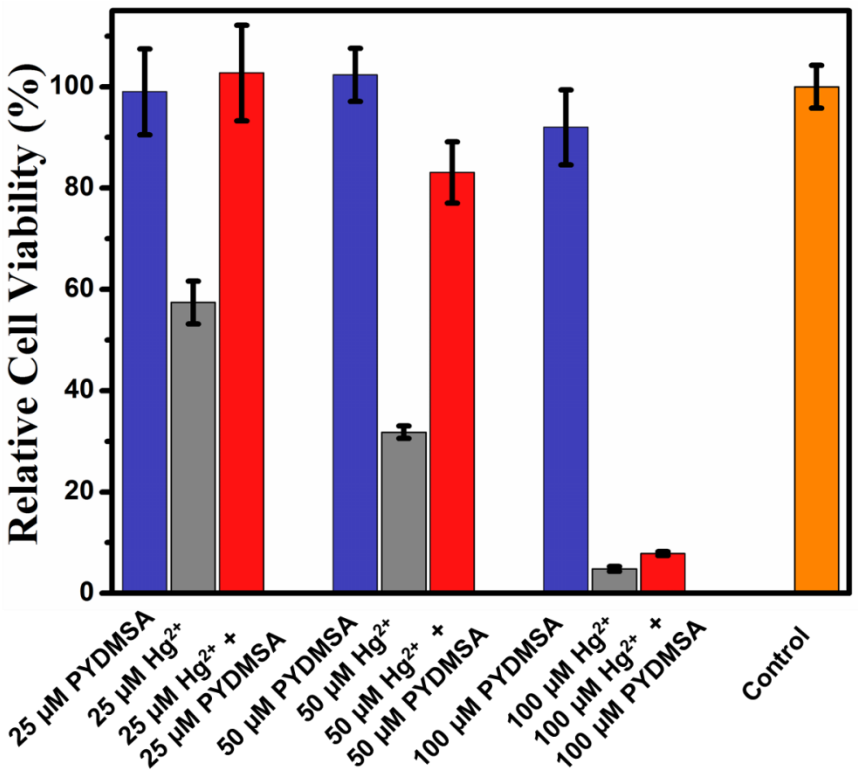


Figure 4. Cell viability assay in the presence of Hg^{2+} and PYDMSA. The bar graphs denote the effect on cell viability when mouse fibroblasts L929 cells were exposed to two different concentrations (25, 50 and 100 µM) of PYDMSA alone, Hg^{2+}alone, and a mixture of $Hg^{2+}$and PYDMSA where cells were pre-incubated with Hg^{2+} for1 h followed by PYDMSA treatment for 24 hrs. The cell viability was assessed colorimetrically using cell counting kit-8 (CCK-8). Cell viability is expressed as a percentage relative to control assuming 100% cell viability for vehicle treated control cells. Values represent mean ± SD.

However, at a higher concentration of Hg^{2+} (100 µM) the cell viability does not get increased much for Hg^{2+} +PYDMSA (4.83 ± 0.48 at 100 µM Hg^{2+}, and 7.81 ± 0.39 for 100 µM Hg^{2+} + PYDMSA). This might be because, for a constant number of cells, 100 µM concentrations become too high to survive. Beside CCK-8 reagent, MTT assay was further carried out for

testing the detoxifying capability of PYDMSA (Figure S12). For assaying the cell viability using MTT, a similar method is used except the absorbance was measured at 570 nm. The trend of cell viability is found to be similar to the assay performed using CCK-8 reagent. In case of MTT assay too, at 25 and 50 μM, toxic effects of Hg^{2+} was substantially neutralized by PYDMSA; but at 100 μM concentration, it shows only 21% viability (Figure S12). However, at every concentration, the cell viability of Hg^{2+} +PYDMSA is higher than that of the only Hg^{2+}. This once again indicates the detoxification capability of PYDMSA. Thus, this work paves the way for studying the dose-determination of chelating agents, and factors which determine the detoxifying properties of sensors like PYDMSA.

CONCLUSIONS

In conclusion, we have developed a next-generation 'turn-on' fluorescent sensor, PYDMSA, which can simultaneously detect and detoxify inorganic mercury in living samples. The sensor, which can be prepared by a simple one-step synthesis from Pyronin Y and DMSA, responds rapidly to Hg^{2+} under physiological conditions in a 1:1 stoichiometry. The distinct color and fluorescence changes due to the CS-C[9] bond cleavage makes this derivative extremely useful for sensing Hg^{2+} ions through fluorescence and/or naked–eye detection in physiological conditions. The experimental detection limit of Hg^{2+} is found to be ~300 pM which is much lower than the standard of contamination in water given by USEPA. The PYDMSA can be used for live cell and *in vivo* monitoring of Hg^{2+} uptake and can detoxify mercury from the samples. This work paves the way for studying the factors determining the detoxifying properties of sensors like PYDMSA. A sensor that can simultaneously detect and detoxify Hg^{2+} in live cell, like PYDMSA, may find widespread application in dose-determination of chelating agent, detection, remediation and understanding precise mechanism of Hg^{2+} induced toxicity in living system.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Characterization of PYDMSA by FTIR spectra; ^{1}H and ^{13}C NMR of PYDMSA; HRMS of PYDMSA; UV-Visible spectroscopic characterization of PYDMSA; Color change of the solution containing PYDMSA in the presence of Hg^{2+}; Time-dependent response of PYDMSA to Hg^{2+}; HRMS of the Hg^{2+}-DMSA complex and pyronin Y; Fluorescence intensity vs. Hg^{2+} concentration plot; Job's plot: PYDMSA vs. Hg^{2+}; Fluorescence titration at nanomolar (nM) concentrations of Hg^{2+}; Response of PYDMSA towards various metal ions; MTT assay for the cell viability test.

AUTHOR INFORMATION

Corresponding Author

(E. P.): pre@iitm.ac.in.

(T. T.): tijuthomas@iitm.ac.in; tt332@cornell.edu

Present Addresses

K.B., Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125 **A.K.D.**, Vienna University of Technology, Institute for Applied Physics - Biophysics group, Getreidemarkt 9, 1060 Vienna, Austria

Author Contributions

$^{\Phi}$ AM and KB contributed equally.

Notes

The authors declare no competing financial interest.

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